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Trichoderma asperellum, an inoculant for the production of steviol glycosides in *Stevia rebaudiana* Bertoni plants micropropagated in a temporary immersion bioreactor

Trichoderma asperellum, un inoculante para la produción de glucósidos de esteviol de plantas de *Stevia rebaudiana* Bertoni micropropagadas en biorreactor de inmersión temporal

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Abstract

Stevia rebaudiana is a plant that synthesizes steviol glycosides, compounds with sweetening properties. The aim of this work was to establish a system for the micropropagation of *S. rebaudiana* plants in a temporary immersion bioreactor (TIB) and evaluate the effect of inoculation with *Trichoderma asperellum* of the micropropagated plants on the plant growth and the accumulation of steviol glycosides and phenolic compounds in leaves. *S. rebaudiana* plants with intact roots were propagated in the TIB with Murashige and Skoog medium and 0.37 mg L^{-1} indolebutyric acid; subsequently, the plants were inoculated with spores of *T. asperellum* (4×10^4 spores mL^{-1}). The plants propagated and inoculated with the fungus exhibited growth significantly larger than that of the uninoculated plants (control). The inoculated plants produced three-fold more steviol glycosides than the uninoculated plants (control), while the phenolic compound contents the inoculated and uninoculated plants were similar. These results encourage the possibility of using a TIB and *T. asperellum* for the propagation and growth promotion of *S. rebaudiana* plants with high steviol glycoside contents.

Keywords: *In vitro* culture, phenolic compounds, biostimulant, natural sweetening compounds, bioreactor.

Resumen

Stevia rebaudiana es una planta que sintetiza glucósidos de esteviol, compuestos con propiedades edulcorantes. El objetivo de este trabajo fue establecer un sistema de micropropagación de plantas de *S. rebaudiana* en un bioreactor de inmersión temporal (TIB) y evaluar el efecto de la inoculación con *Trichoderma asperellum* de las plantas propagadas en el crecimiento, en la producción de glucósidos de esteviol y compuestos fenólicos. Las plantas de *S. rebaudiana* con raíces se propagaron en el TIB con el medio Murashige y Skoog con 0.37 mg L^{-1} de ácido indolbutírico; posteriormente, las plantas propagadas se inocularon con esporas de *T. asperellum* (4×10^4 esporas mL^{-1}). El crecimiento de las plantas propagadas e inoculadas con el hongo fue significativamente mayor que el de las plantas no inoculadas con el hongo (control). Las plantas inoculadas produjeron tres veces más glucósidos de esteviol que las plantas no inoculadas, mientras que el contenido de compuestos fenólicos fue similar. Estos resultados muestran la posibilidad de usar los sistemas TIB y la inoculación con *T. asperellum* para la propagación y promoción del crecimiento de plantas de *S. rebaudiana* con alto contenido de glucósidos de esteviol.

Palabras clave: Cultivo *in vitro*, compuestos fenólicos, bioestimulante, compuestos edulcorantes naturales, biorreactor.

1 Introduction

Stevia rebaudiana (Bertoni) is a perennial shrub belonging to the family Asteraceae, and it is native to the tropical region of Amambay-Paraguay. The leaves of *S. rebaudiana* contain more than 30 steviol glycosides and phenolic compounds of nutritional and pharmacological interest (Lemus-Moncada *et al*., 2012; Ceunen and Geuns 2013). Purified steviol glycosides are 300-400-fold sweeter than sucrose and are used as noncaloric sweeteners.

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Thus, steviol glycosides are an excellent sweetening alternative for overweight and diabetic patients (Wölwer-Rieck 2012; Shivanna *et al*., 2013). The most abundant steviol glycosides are stevioside (6-10%) and rebaudioside A (2-4%) (Morlock *et al*., 2014).

S. rebaudiana plants are mainly propagated with cuttings; however, the plants propagated with this method show variable steviol glycoside contents, and alternative methods of propagation are required. Plant tissue culture is recognized as a powerful tool for the clonal propagation of plants. In particular, the use of temporary immersion bioreactors (TIB) is a preferred method for the use of classical propagation systems in semisolid media. TIB systems are automated and improve nutrient availability and gas transfer, preserve morphological integrity and favor the rapid growth of plants (Welander *et al*., 2014). The plants produced in TIB show increased biomass yield, a higher photosynthetic rate, decreased hyperhydricity and larger size. In several plant species, the production of secondary metabolites is increased (Arencibia *et al*., 2008). Studies of *S. rebaudiana* propagation and their production of compounds of interest in TIB have been scarce. Alvarenga and Salazar (2015) showed that stevia plants grown in TIB systems reached greater lengths and higher bud numbers per explant compared to those grown in semisolid medium.

Inoculation with beneficial microorganisms during plant micropropagation may improve crop quality (Botta *et al*., 2013; Pérez-Montaño *et al*., 2014). The inoculation of beneficial microorganisms promotes plant growth, improves nutrient-use efficiency (Meena *et al*., 2017), and increases the contents of secondary metabolites, for example, flavonoids and phenolic compounds (Ortega-García *et al*., 2015). In relation to *S. rebaudiana* plants, the bacteria *Burkholderia gladioli, Enterobacter aerogenes*, and *Serratia marcescens* (Mamta *et al*., 2010) and the mycorrhizal fungus *Rhizoglomus irregulare* (Tavarini *et al*., 2018) promote growth and production of steviol glycosides. Inoculation with the fungus *Piriformospora indica* and the bacterium *Azotobacter chroococcum*, alone or in combination, improves the growth and antioxidant activity of *S. rebaudiana* cultured *in vitro* and in semisolid medium (Kilam *et al*., 2015). However, to the best of our knowledge, the beneficial effect of inoculation with fungus of the genera *Trichoderma* in *S. rebaudiana* plants has not yet been studied. *Trichoderma* is a fungus that is nonpathogenic to humans and has biotechnological value due its ability to grow under diverse environmental conditions. In crops of interest, *Trichoderma* promotes plant growth and functions as a biological control agent (Mukherjee *et al*., 2012). Additionally, Sofo *et al*., (2012) showed that the *in vitro* inoculation of plants with *T. harzianum* increases plant survival during the acclimatization phase in the greenhouse.

Previously, we reported that the isolate TC3 of *T. asperellum* promotes the growth of two varieties of onion (*Allium cepa* L.) plants. The growth promotion is related to the ability of *T. asperellum* to produce indol-3-acetic acid (IAA), siderophores and to solubilize phosphate (Ortega-García *et al*., 2015). In this work, we established a system for the micropropagation of *S. rebaudiana* plants in TIB and evaluated the effect of inoculation with *T. asperellum* on the growth and accumulation of steviol glycosides and phenolic compounds in the leaves of the propagated plants.

2 Materials and methods

2.1 Plant material and *Trichoderma asperellum*

S. rebaudiana plants propagated *in vitro* were provided by Silvana Alvarenga-Venutolo from Instituto Tecnológico de Costa Rica. The *in vitro* cultures were maintained according to the method of Oviedo *et al*., (2015). The *T. asperellum* To isolate was obtained from Allium cepa L. grown in the Morelos state of Mexico and has been registered under GenBank KP059112 (Ortega-García 2015).

2.2 Temporary immersion bioreactor systems (TIB)

The TIB system (Fig. 1) was implemented using two 1 L flasks, as described in Oviedo *et al*. (2015). The plants were placed in flask A, while flask B was used as a medium reservoir; the flasks were connected with a 25-gauge silicone pipe (Master Flex). Two adapters were placed in each flask (Thermo Scientific), and a 0.22-μm pore-size filter (Whatman) was connected. The air was supplied with an oil-free compressor (Poterclable), which generates a pressure of 20 psi, and the aeration system was set at 2.7 volumes of air/medium volume/min (vvm). The immersion frequency of the system was 10 min every 12 h, and it was controlled with a programmable timer unit (Siemens) and two solenoid valves (Ascoy).

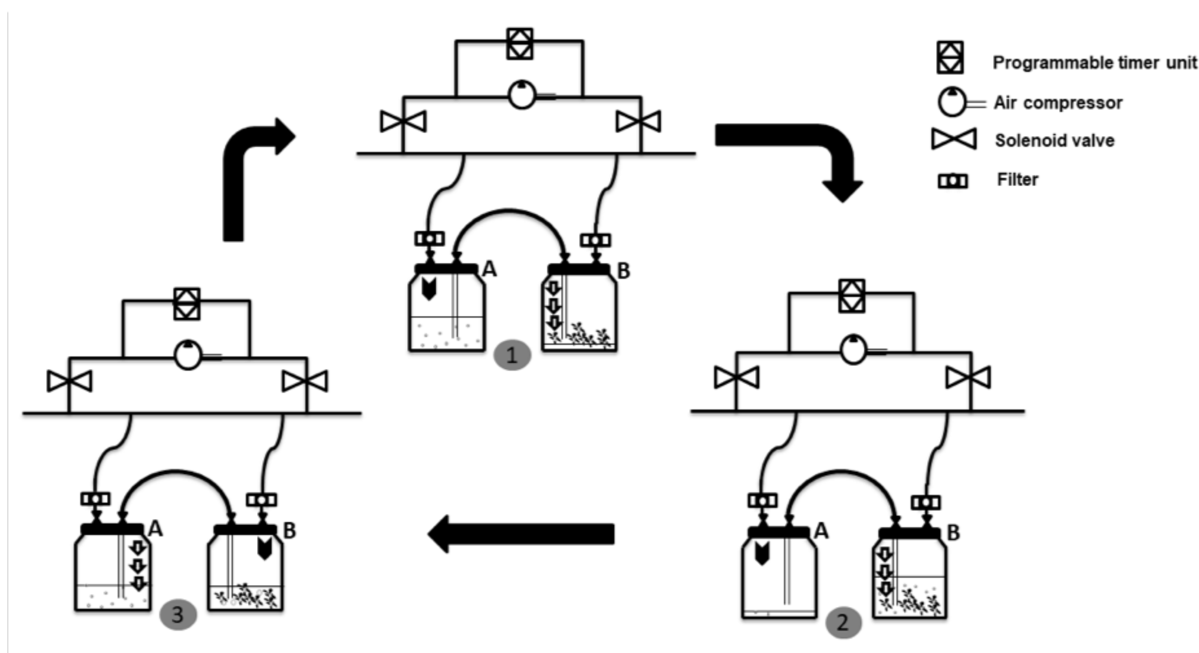

Fig. 1. Temporary Immersion Bioreactor (TIB) cycle diagram: 1 Flask of reservoir medium (A), and plant culture flask (B); 2 Air pressure allows the passage of the culture medium (A) to flask with explants (B) and the immersion of the explants in flask; 3 Air pressure at reverse flow returns the culture medium from flask B to flask A.

The TIB system was maintained at 25 ± 3 °C, with a photoperiod of 16 h light/8 h dark, and daylight was simulated using an LED strip at a light intensity of 11.14 μmol m^{-2} s^{-1} PPFD.

2.3 Effect of plant growth regulators (PGR) in the propagation of Stevia rebaudiana in TIB

Internodal segments (10 pieces) of *S. rebaudiana* plants propagated *in vitro* were placed in each TIB with 300 mL of MS medium (Murashige and Skoog 1962) supplemented with 0.5 mg L^{-1} gibberellic acid (GA$_3$), 30 g L^{-1} sucrose and 2 mg L^{-1} calcium pantothenate at a pH of 5.7. To obtain complete rooting of plants, the following plant growth regulators (PGRs) were added to the culture media: 0.37 mg L^{-1} indol-3-butyric acid (IBA); 0.25 mg L^{-1} indoleacetic acid (IAA); 0.1 mg L^{-1} IBA + 0.25 mg L^{-1} IAA; and 0.1 mg L^{-1} IBA + 0.25 mg L^{-1} 6-benzylaminopurine (BAP). The control consisted of plants not treated with PGR. The plants were collected after 37 days of culture, and the number of buds and leaves, plant height, and plant and root length were recorded. The plants propagated in TIB that developed roots were selected for the acclimatization phase in pots.

2.4 Inoculation with T. asperellum of S. rebaudiana plants propagated in TIB

The To isolate of *T. asperellum* was grown for 15 days in Potato Dextrose Agar (PDA, Bioxon) medium and was prepared for the spore suspension. Two spore concentrations (4 $\times 10^4$ and 3 $\times 10^6$ spores mL^{-1}) of *T. asperellum* and two exposure times (3 and 6 days) were tested for inoculation of *S. rebaudiana* plants in the TIB system. The highest survival of plants (69%) was obtained with the concentration of 4 $\times 10^4$ spores mL^{-1} and the exposure time of 3 days. For other treatments, the plant survival was less than 20%. Thus, a suspension of 4 $\times 10^4$ spores mL^{-1} for 3 days of exposure was chosen as the inoculation condition for *S. rebaudiana* plants grown for 42 days in a TIB system. *S. rebaudiana* plants grown in a TIB system without *T. asperellum* inoculation were the control.

2.5 Growth of S. rebaudiana plants in pots

Plants inoculated (TB) and uninoculated (Control, CT) with *T. asperellum* in TIB were transplanted in 1 L pots which measured 11 cm in height and 10 cm in diameter. The substrate utilized was a mixture

composed of 60% peat moss, 20% perlite, and 20% vermiculite, and it had a pH of 5.6 ± 0.5 and 85% porosity. The assay was carried out from September 2015 to January 2016 at an average temperature of 22 °C, a maximum temperature of 29 °C, a minimum temperature of 15 °C, and a relative humidity of 62%. The plants were grown for 53 days, and the number of buds and leaves, plant height, root length, stem diameter, fresh weight (FW), and dry weight (DW) were recorded. The steviol glycoside and phenolic compounds in the leaves was measured.

2.6 Steviol glycoside quantification by High Performance Thin Layer Chromatography (HPTLC)

The fresh leaf tissue (0.1 g) was ground in a mortar with liquid nitrogen and suspended in 1 mL of methanol (J.T. Baker, New Jersey, United States) following the methodology of Wagner and Bladt (1996). The mixture was stirred for 3 min, allowed to stand for 24 h without stirring, and then centrifuged at 10,000 rpm at 4 °C for 10 min. The supernatant was recovered, placed in Eppendorf tubes, and stored at -4 °C until the analysis by HPTLC. Determination of steviol glycosides was performed based on the methodology reported by Bladt and Zgainski (1996) and Morlock *et al*. (2014). Stevioside (Sigma-50956), Rebaudioside A Reb-A (ASB-00018223-002), Rebaudioside C Reb-C (ASB-00018228-005), and Dulcoside A Dul-A (ASB-00004949-005) were utilized as standards. HPTLC plates of 20 x 10 cm of silica gel 60 F254 (Merck, Darmstadt, Germany) were used. The samples were applied to the plates with an automatic TCL sampler ATS4 (CAMAG, Muttenz, Switzerland). The compounds in the plates were separated with the solvent system consisting of a 65:25:4 (v:v:v) mix of chloroform: methanol: acetic acid. The HPTLC plates were revealed by spraying with α-naphthol, which was generated by dissolving 2 g of α-naphthol in 180 mL of absolute ethanol and 12 mL of 50% H_2SO_4 in water. The plates were heated at 120 °C for 5 min, and the digitized image was recorded under white light. Quantification of steviol glycosides was carried out using the VisionCATS ver. 2.0.15069.1 program. The calibration curve was constructed using steviol-glycoside standards (0.2-2.0 μg). The obtained Rf values were as follows: Dul-A (0.29); Stevioside (0.19); Reb-C (0.15), and Reb-A (0.12).

2.7 Determination of phenolic compounds in S. rebaudiana plants propagated in TB

The dry tissue of leaves was extracted with 75% ethanol at a ratio of 1:10 (dry tissue: solvent). The mixture was centrifuged at 10,000 rpm at 4 °C for 10 min, and the supernatant was recovered. The total phenolic compounds in the ethanolic extracts were determined using the Folin-Ciocalteu reagent according to the methodology reported by Bobo-García *et al*. (2015). Gallic acid was used as standard to generate a standard curve (0-100 μg mL^{-1}). The assay was performed on a microplate, and the optical density was measured at a wavelength of 760 nm on a MultiskanGo brand plate reader equipped with SkanIt ver. 1.00.40 software (Thermo Fisher Scientific, Massachusetts, USA). The results were expressed as mg of Gallic Acid Equivalents (GAE) g^{-1} DW.

2.8 Statistical analysis

The results were analyzed in the InfoStat version 2016 statistical software program, and each variable was tested for normality (Shapiro-Wilks). An analysis of variance (ANOVA) was performed to find significant differences among the means. Tukey (parametric) and Kruskal-Wallis (nonparametric) mean comparison tests were performed with a significance level of $\alpha = 0.05$.

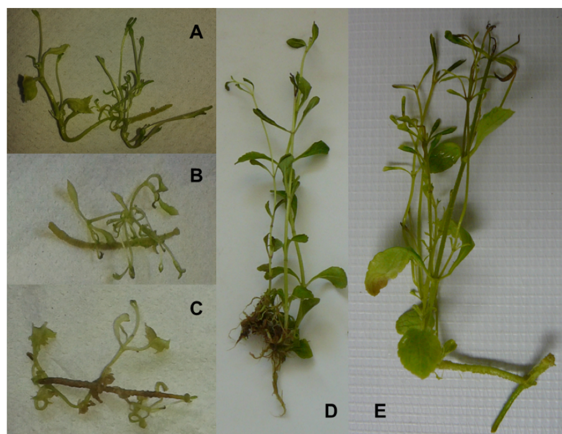

Fig. 2. Plants of *Stevia rebaudiana* grown in a temporary immersion bioreactor with different treatments of plant growth regulators. A. 0.25 mg L^{-1} IAA; B. 0.1 mg L^{-1} IBA + 0.25 mg L^{-1} IAA; C. 0.1 mg L^{-1} IBA + 0.25 mg L^{-1} BAP; D. 0.37 mg L^{-1} IBA; and E. Control.

Table 1. Development of *S. rebaudiana* plants with different treatments of plant growth regulators (PGR) in a temporary immersion bioreactor.

PGR	Concentration (mg L^{-1})	Buds number	Leaves number	Plant height (cm)	Roots lenght (cm)
Control	0	5.8 ± 0.2^a	34.6 ± 3.2^b	14.9 ± 1.6^b	-
IBA	0.37	14.0 ± 0.6^c	44.0 ± 5.6^b	13.9 ± 1.3^b	1.3 ± 0.3
IAA	0.25	11.2 ± 0.7^b	14.0 ± 3.5^a	6.4 ± 0.6^a	-
IBA+IAA	0.1+0.25	6.2 ± 0.8^a	11.0 ± 1.9^a	4.0 ± 0.3^a	-
IBA+BAP	0.1+0.25	4.4 ± 0.6^a	13.4 ± 1.6^a	4.3 ± 0.4^a	-

Data are means ± standard error. The values with different letter are significantly different (p >0.05)

3 Results and discussion

S. rebaudiana plants with intact roots and leaves without malformations were grown in the TIB system supplemented with 0.37 mg L^{-1} IBA (Fig. 2D). The plants had a mean of 14.0 buds, 44 leaves, total length of 13.9 cm, and principal root length of 1.3 cm (Table 1). The plants obtained with the IAA treatment generated 11.2 buds and normal leaves but no roots (Fig. 2A), while plants treated with combinations of IBA + IAA and IBA + BAP had the lowest number of buds, no roots, and leaf malformations (Fig. 2B, C).

The *S. rebaudiana* plants propagated in the TIB system and inoculated with *T. asperellum* (TB plants) had a survival of 69%, while the survival of the uninoculated plants (CT plants) was 75%. Fig. 3 shows the appearance of *S. rebaudiana* plants grown for 53 days in pots. The TB plants grown in the TIB system exhibited better growth than the CT plants. The plant height, bud number, leaf area, and fresh weight were significantly greater in TB plants than in CT plants (Table 2).

S. rebaudiana is a plant with great commercial potential due to its sweetening properties, and effective propagation systems are necessary to improve its yield and taste qualities (Botta *et al*., 2013; Kilam *et al*., 2015). *In vitro* micropropagation systems are a possible for obtaining clonal plants from mother plants that have been selected for their outstanding quality. Although there have been micropropagation studies of *S. rebaudiana* plants using semisolid media (Thiyagarajan and Venkatachalam 2012; Singh *et al*., 2014) and liquid media (Alvarenga and Salazar 2015), the plants generated lacked roots. Consequently, it had been necessary to implement an additional stage for the successful rooting of the plants. In this work, the use of TIB with 0.37 mg L^{-1} IBA facilitated the generation of plants with roots which may adapt easier to the growing conditions in pots. Similarly, the TIB systems have been used as a viable alternative for the micropropagation of other plant species of interest. Kunakhonnuruk *et al*. (2019) compared three micropropagation systems of *Drosera communis* and showed that the TIB system was the most suitable method for large-scale production of biomass and the compound of interest (plumbagin). Ramírez-Mosqueda *et al*. (2016) compared three different bioreactor systems in two different micropropagation phases (multiplication and rooting) for the propagation of *Vanilla planifolia*; the authors confirmed the utility of BIT systems in the commercial micropropagation of this species and that this system may reduce the costs of use of systems other. Additionally, Arencibia *et al*. (2008) showed that sugarcane plants (*Saccharum officinarum*) micropropagated in TIBs with high levels of phenolic compounds displayed an increase in roots number, growth rate and ability to be colonized by the endophytes (*Gluconacetobacter diazotrophicus*) during adaptation to natural conditions.

Our results show that the micropropagation of *S. rebaudiana* plants in TIB and inoculation with *T. asperellum* produces larger plants with a greater number of shoots and foliar area. The effect of the *Trichoderma* fungi on plant growth promotion is widely known; this plant growth promotion is related to the roles of *Trichoderma* in the production of compounds similar to indolacetic acid (AIA), the ability to solubilize phosphates and produce compounds named siderophores, and ability to enhance nutrient availability (Kashyap *et al*., 2017; Cai *et al*., 2015). Thus, the growth promotion of *S. rebaudiana* plants may related with the ability of *T. asperellum* to produce AIA and solubilize phosphates (Ortega-García *et al*., 2105). To the best of our knowledge, this is the first study on the use of *T. asperellum* to promote growth of *S. rebaudiana* plants.

Table 2. Growth parameters of *S. rebaudiana* plants propagated in a temporary immersion bioreactor and inoculated with *T. asperellum*, 53 days after transplant at pots.

Treatment	Plant height (cm)	Buds number	Leaf area (cm^2)	Weight (g) Fresh	Weight (g) Dry	Stem diameter (cm)	Root lenght (cm)	Leaf number
CT plants	12.6 ± 0.6^b	7.6 ± 0.4^b	29.0 ± 3.9 ^{b}	2.0 ± 0.1^b	0.24 ± 0.02 ^{a}	0.12 ± 0.01^a	19.0 ± 0.9^a	53.4 ± 4.8^a
TB plants	20.6 ± 1.6^a	12.8 ± 0.7^a	58.9 ± 12.4^a	2.8 ± 0.1^a	0.30 ± 0.01 ^{a}	0.18 ± 0.02^a	19.6 ± 1.3^a	73.4 ± 11.2^a

CT, control plants; TB, plants inoculated with *T. asperellum.*
Data are means ± standard error. The values with different letter are significantly different (p >0.05)

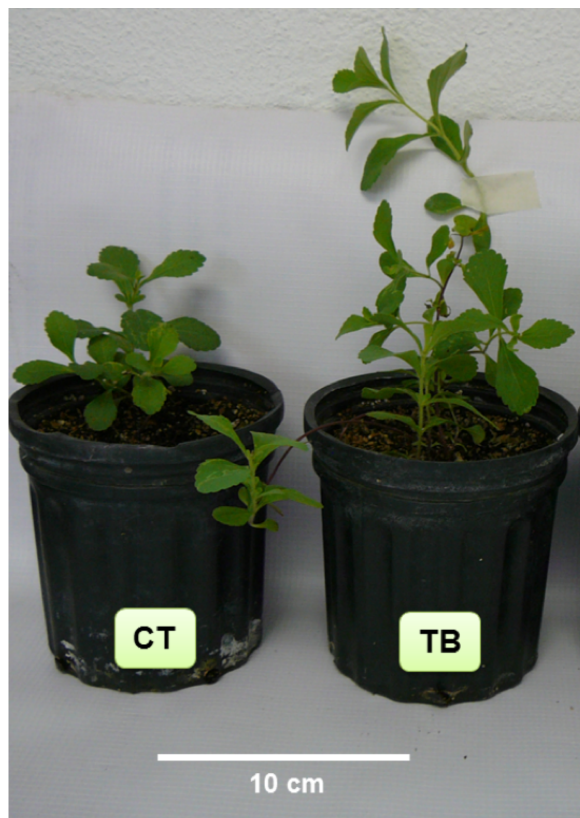

Fig. 3. Plants of *Stevia rebaudiana* generated in a temporary immersion bioreactor and non-inoculated (CT) and inoculated with *Trichoderma asperellum* (TB). The plants were evaluated after 53 days of transplant at pots.

The content of total steviol glycosides, stevioside and Reb-A were three-fold greater in TB plants than in CT plants (Table 3). The steviol glycoside content is consistent with those reported by other authors in plants inoculated with bacteria (Mamta *et al*., 2010) and mycorrhizal fungus (Tavarini *et al*., 2018). However, the plants treated with *T. asperellum* in the TIB system showed a decrease in the Stevioside/Reb-A ratio relative to the control (Table 3). This result suggests that inoculation with *T. asperellum* may modulate the synthesis of different steviol compounds.

The content of phenolic compounds of the TB plants were similar to those of the CT plants (Table 4). These results indicate that inoculation with *T. asperellum* does not affect the content of phenolic compounds in *S. rebaudiana* plants. In contrast, *T. asperellum* modulates the accumulation of phenolic compound and flavonoids in two varieties of onion plants grown in greenhouse conditions (Ortega-García *et al*., 2015). This suggests that the effect of *T. asperellum* on the synthesis of phenolic compounds depends on the plant species and environmental conditions. In this study, inoculation of *S. rebaudiana* plants with *T. asperellum* was under *in vitro* conditions. Alternatively, nitrogen reduction in a TIB system may enhance the production of phenolic compounds in *Castilleja tenuiflora* (Cortes-Morales *et al*., 2018). Research in cell suspension cultures of *Buddleja cordata* reported a phenolic compound content of 87.4 mg DW^{-1} (Gutiérrez-Rebolledo *et al*., 2018).

Table 3. Content of steviol glycosides of *S. rebaudiana* plants obtained in a temporary immersion bioreactor.

Treatment	Total of steviol glycoside	Stevioside (mg DW^{-1} leaf)	Reb-A	Dul-A	Stevioside/Reb-A
CT plants	5.0± 2.3^b	4.4 ± 2.0^b	0.3 ± 0.13^b	0.4 ± 0.10^a	14.6
TB plants	15.6 ±1.8^a	13.8 ± 1.7^a	1.1 ± 0.11^a	0.6 ± 0.08^a	12.5

CT, control plants; TB, plants inoculated with *T. asperellum.*
Data are means ± standard error. The values with different letter are significantly different (p >0.05)

Table 4. Content of phenolic compounds in *S. rebaudiana* plants obtained in a temporary immersion bioreactor.

Treatment	Phenolic compounds (mg GAE DW^{-1} leaf)
CT plants	4.44 ± 0.8^a
TB plants	3.91 ± 0.8^a

CT, control plants; TB, plants inoculated with *T. asperellum.*

Data are means ± standard error. The values with different letter aresignificantly different (p >0.05).

Conclusions

The TIB system and the use of *T. asperellum* as an inoculant is an alternative for the micropropagation of *S. rebaudiana* plants. It enables better growth and higher steviol glycoside content for plants growing in pots.

Acknowledgements

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